Original Article

DOI: 10.5582/bst.2015.01092

Products of dentin matrix protein-1 degradation by interleukin-1β-induced matrix metalloproteinase-3 promote proliferation of odontoblastic cells

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Summary **We have previously reported that interleukin (IL)-1β induces matrix metalloproteinase (MMP)-3-regulated cell proliferation in mouse embryonic stem cell (ESC)-derived odontoblast-like cells, suggesting that MMP-3 plays a potentially unique physiological role in regeneration by odontoblast-like cells. MMPs are able to process virtually any component of the extracellular matrix, including collagen, laminin and bioactive molecules. Because odontoblasts produce dentin matrix protein-1 (DMP-1), we examined whether the degraded products of DMP-1 by MMP-3 contribute to enhanced proliferation in odontoblast-like cells. IL-1β increased mRNA and protein levels of odontoblastic marker proteins, including DMP-1, but not osteoblastic marker proteins, such as osteocalcin and osteopontin. The recombinant active form of MMP-3 could degrade DMP-1 protein but not osteocalcin and osteopontin *in vitro*. The exogenous degraded products of DMP-1 by MMP-3 resulted in increased proliferation of odontoblast-like cells in a dose-dependent manner. Treatment with a polyclonal antibody against DMP-1 suppressed IL-1β-induced cell proliferation to a basal level, but identical treatment had no effect on the IL-1β-induced increase in MMP-3 expression and activity. Treatment with siRNA against MMP-3 potently suppressed the IL-1β-induced increase in DMP-1 expression and suppressed cell proliferation ($p < 0.05$). Similarly, treatment with siRNAs against Wnt5a and Wnt5b suppressed the IL-1β-induced increase in DMP-1 expression and suppressed cell proliferation ($p < 0.05$). Rat KN-3 cells, representative of authentic odontoblasts, showed similar responses to the odontoblast-like cells. Taken together, our current study demonstrates the sequential involvement of Wnt5, MMP-3, DMP-1 expression, and DMP-1 degradation products by MMP-3, in effecting IL-1β-induced proliferation of ESC-derived odontoblast-like cells.**

Keywords: Embryonic stem cell, odontoblast, Wnt5

1. Introduction

Because matrix metalloproteinases (MMPs) are able to process virtually any component of the extracellular matrix (ECM), including collagen, laminin, and bioactive molecules, it has been suggested that MMPs may be important in inflammatory conditions, such as rheumatoid arthritis, metastasis and periodontitis (*1,2*). In particular, MMP-3 (known as stromelysin-1) has a wide substrate specificity and is capable of degrading many types of ECM proteins, such as collagen types II, III, IV, IX, and X, proteoglycans, fibronectin, laminin, and elastin (*1,2*), rendering MMP-3 crucial in connective tissue and bone remodeling (*3,4*). However, while it is intuitive that dental pulp destruction may be a function of MMPs, our previous study reported that MMP-3 actually accelerates wound healing following dental pulp injury (*5,6*). This observation indicates that MMP-3 may instead be involved in ECM

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degradation, especially in dentin, and in the subsequent morphogenesis, wound repair (*5,6*) and angiogenesis (*5-7*) of the inflamed tissue.

Interleukin (IL)-1β detected in inflamed dental pulp, is associated with periapical disease (*8*) and is apparently essential to the pathogenesis of acute pulpitis. Notably, treatment with IL-1β induces potent expression of *MMP-3* in dental pulp, a tissue that contains large numbers of odontoblasts (*7*). We also reported that proinflammatory cytokine IL-1β-induced MMP-3 activity is associated with cell proliferation of purified odontoblast-like cells derived from mouse embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) (*9,10*). Thus, IL-1β-induced MMP-3 appears to be pivotal in the pathophysiology of inflamed dental pulp containing rich odontoblasts. However, the signaling cascade underpinning this stimulation is yet to be elucidated.

Because performing experiments using purified odontoblast-like cells derived from mouse iPSCs (*11*) and ESCs (*12*), we have developed excellent *in vitro* models in which to examine the mechanisms of wound healing following exposure to proinflammatory cytokines (*9,10*). We have also previously reported that a proinflammatory cytokine mixture of IL-1β, tumor necrosis factor-α, and interferon-γ, or IL-1β alone, can induce MMP-3 activity in rat dental pulp cells, and result in a potent increase in cell proliferation (*13,14*). Taken together, these studies suggest that MMP-3 induced by the proinflammatory cytokine IL-1β contributes to the pathophysiology of inflamed dental pulp containing rich odontoblast cells.

It is unclear how IL-1β cytokine-induced MMP-3 regulates odontoblast-like cell proliferation. Recent reports demonstrate that IL-1β-induced MMP-3 is associated with the secreted glycoprotein Wnt signaling pathway (*15*). We have also demonstrated that IL-1β-induced MMP-3-regulated proliferation of mouse ESC-derived odontoblastic cells is mediated by the Wnt5a- and 5b-signaling pathways (*16*). Although compelling evidence remains to be shown, we speculate that an ECM-derived degradation product generated by MMP-3 might induce cell proliferation; however, the substrates of MMP-3 that induce cell proliferation remain to be identified.

Odontoblasts are essentially dentin-secretory cells that produce pre-dentin, an ECM formed by type I collagen as the major organic component (about 90%), together with noncollagenous proteins, including glycoproteins, proteoglycans, and dentin phosphoproteins, such as dentin matrix protein-1 (DMP-1), which constitute relatively specific markers for dentin (*17,18*). Therefore, odontoblasts are surrounded by collagen and dentin matrix proteins. A recent report has demonstrated that ECM, especially the dentin matrix protein component, contains functional proteins that have been previously defined as solely intracellular (*19*). However, DMP-1, which has a role in matrix mineralization, can act as both an intra- and extracellular-signaling molecule, thus impacting odontoblast differentiation (*20,21*). To date, functional data, especially in respect to the proliferative roles of MMP-3 signaling and DMP-1 in mouse odontoblasts, remains scarce. Therefore, further studies are required to completely understand the intracellular role of DMP-1 in odontoblasts.

Here, we examine whether DMP-1 degradation products by MMP-3 are associated with the proliferation of odontoblasts, as may be encountered in inflamed dental pulp. We show for the first time, that MMP-3 up-regulates dentin matrix protein degradation in our odontoblast-like cells, and leads to increased cell proliferation.

2. Materials and Methods

2.1. *Products*

Mouse recombinant IL-1β was obtained from PeproTech (Rocky Hill, NJ, USA). Mouse recombinant DMP-1 and osteopontin were obtained from R&D Systems Inc. (Minneapolis, MN, USA) and osteocalcin was obtained from Uscn Life Science Inc. (Wuhan Hubei, China). Anti-DMP-1 polyclonal antibody was obtained from Abcam (ab103203; Cambridge, UK). Recombinant mouse MMP-3 (Pro-form) was obtained from R&D Systems Inc., and was activated by treating with 1 mM 4-aminophenyl mercuric acetate in all experiments.

2.2. *Cell culture*

The mouse ESC cell line, E14Tg2a (*22*), was a kind gift from Dr. Randall H. Kramer (University of California San Francisco, San Francisco, CA, USA) and was maintained as described previously (*23*). Purified ESC-derived odontoblast-like cells were obtained as previously reported (*12*). These differentiated cells displayed odontoblast-like physiological characteristics, for example calcification activity and alkaline phosphatase activation, up to day 21 of culture. Authentic rat odontoblast-like cells (KN-3) (*24*) were kindly provided by Dr. Chiaki Kitamura (Kyushu Dental College, Kitakyushu, Japan) and were maintained as described previously (*24*). Mouse osteoblast-like MC3T3-E1 cells were obtained from the Riken BioResource Center Cell Bank (Ibaraki, Japan) and were cultured as previously described (*25,26*). These cells were used throughout the study as a negative control.

2.3. *Cell proliferation assay*

Cell proliferation was evaluated using the BrdU-cell proliferation enzyme-linked immunosorbent assay (ELISA; Roche Applied Science, Mannheim, Germany) as described previously (*25,26*). Cells were seeded

into 96-well tissue culture plates at a density of 1×10^5 cells/cm^2.

2.4. *Real-time quantitative polymerase chain reaction analysis*

Real-time quantitative polymerase chain reaction (qPCR) for all samples and standards was performed in triplicate in 96-well optical microtiter plates with ~25 ng of RNA, 0.25 μL of RT Mix (Qiagen Quantitect RT Mix), 1.25 μL of 20× Primer/Probe Mix, and 12.5 μL of Mastermix (Qiagen Quantitect RT-PCR Kit) in a 25 μL reaction volume. The following primer/probe sets were used: mouse DMP-1, Mm01208363_m1; rat DMP-1, Rn01450122_m1; human osteocalcin [*BGLAP*] (mouse available), Hs01587814_g1; rat osteocalcin [*BGLAP*], Rn00566386_g1; mouse osteopontin [*SPP1*], Mm00436767_m1; rat osteopontin, Rn00681031_m1; Assays-On-Demand™ (Applied Biosystems, Carlsbad, CA, USA). Standards and samples were mixed with the PCR reagents, loaded into the 96-well microtiter plate and sealed with optical film (Applied Biosystems). TaqMan samples were subjected to thermal cycling conditions with the following parameters: an initial holding stage of 30 min at 50°C (for RNA reverse transcription), 15 min at 95°C (to activate HotStarTaq polymerase enzyme), then 40 cycles of 15 s at 94°C/60 s at 60°C. Gene expression was quantified relative to a standard curve. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 18S amplicon rRNA were employed as housekeeping genes and used as normalization controls to account for variations in the amount of total RNA in each sample. For each experimental sample, the amount of target and endogenous reference was determined from the appropriate standard curve. The amount of target was then divided by the amount of endogenous reference to obtain a normalized target value. Ct (threshold cycle values) for samples and housekeeping genes were extrapolated from the standard curve to produce an arbitrary value of expression, the ratio of which (sample/housekeeping gene) within a given tissue sample was plotted as the relative mRNA expression level.

2.5. *Western blot analysis*

DMP-1, MMP-3, Osteocalcin, Osteopontin, Wnt5a, and Wnt5b protein levels in the cell lysate were determined by western blot analysis. Cells were cultured for 24 h with or without IL-1β, lysed, and the protein lysate separated on sodium dodecyl sulfate polyacrylamide gels (12%). Western blot analysis was then performed using anti-DMP-1 (ab103203; Abcam), anti-MMP-3, anti-osteocalcin, anti-osteopontin, anti-Wnt5a, anti-Wnt5b, and anti-β-tubulin polyclonal antibodies (sc-6839, sc-18322, sc-10593, sc-365370, sc-109464, and sc-9935, respectively; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). The anti-MMP-3 antibody showed no significant cross-reactivity with other MMPs (data not shown). Visualization and quantification of blotted protein bands were performed with Multi Gauge-Ver3.X software (Fujifilm, Tokyo, Japan).

2.6. *Measurement of MMP-3 activity*

The protocol for measuring MMP-3 activity has been described previously (*10,13,16,27*) and is now a commercially available MMP-3 activity assay kit (SensoLyte™ 520 MMP-3 assay kit; AnaSpec, San Jose, CA, USA). Prior to detection, MMP-3 was immunoprecipitated from the culture medium using a goat anti-MMP-3 antibody (sc-6839, Santa Cruz Biotechnology Inc.) and protein A/G–agarose for 6 h at 4°C. After centrifugation, the agarose pellets were suspended in an MMP-3 assay buffer containing the MMP-3 substrate, 5-FAM-Arg-Pro-Lys-Pro-Val-Glu-Nva-Trp-Arg-Lys- QXL™520-NH_2 fluorescence resonance energy transfer peptide, as supplied in the assay kit. MMP-3 activity was then determined according to the manufacturer's instructions (*28*).

2.7. *Silencing of Wnt5a, Wnt5b and MMP-3 genes by siRNA transfection*

The Wnt5a, Wnt5b and MMP-3 siRNAs for gene silencing were acquired commercially (sc-41113, sc-155357 and sc-37265, respectively; Santa Cruz Biotechnology Inc.) and transfected into cultured cells using a siRNA reagent system (Santa Cruz Biotechnology Inc.) according to the manufacturer's protocol. An anti-GAPDH siRNA and an anti-control siRNA, with no known homogeny for any vertebrate sequence (Thermo Scientific, Lafayette, CO, USA), were used as positive and negative controls, respectively.

2.8. *Statistical analysis*

Data presented in bar graphs are the mean ± standard deviation (S.D.) of four independent experiments. Statistical significance was assessed using the Mann-Whitney *U*-test. A value of $p < 0.05$ was considered as statistically significant.

3. Results

3.1. *IL-1β induction of DMP-1 mRNA and protein expression*

The ESC-derived odontoblast-like cell line was cultured in the presence of four concentrations of IL-1β (0, 0.25, 2.5, and 25 ng/mL). Induction of DMP-1, osteocalcin, and osteopontin mRNA and protein were assessed using real-time qPCR (Figures 1A-1C) and western blot analysis (Figures 1A-1C, far lower panels), respectively. mRNA and protein levels of the odontoblastic marker

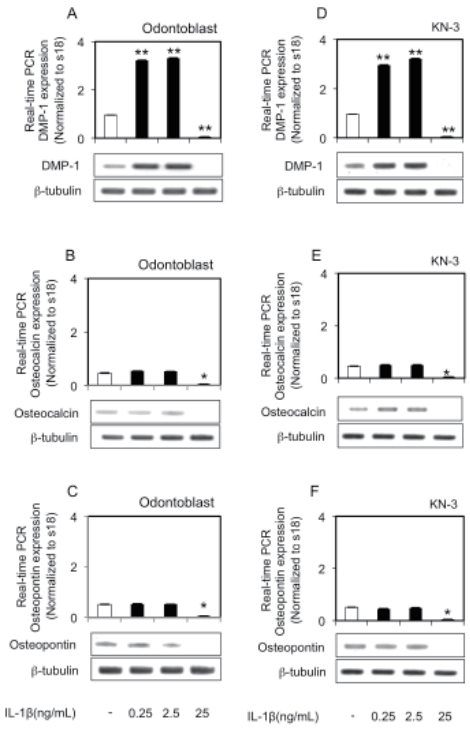

Figure 1. IL-1β-induced expression of DMP-1, osteocalcin, and osteopontin mRNA and protein in odontoblast-like cells. ESC-derived odontoblast-like cells and KN-3 odontoblast cells were incubated with IL-1β (0, 0.25, 2.5, and 25 ng/mL). Real-time qPCR expression of DMP-1, osteocalcin and osteopontin mRNA relative to control (S18 mRNA) using ESC-derived odontoblast-like cells (A-C) and KN-3 cells (D-F). Data represent the mean ± S.D. of four independent experiments. *$p < 0.05$, **$p < 0.01$. Lower panels show western blot analysis of DMP-1, osteocalcin, osteopontin, and β-tubulin protein levels following stimulation with IL-1β. Blots shown are representative of three independent experiments.

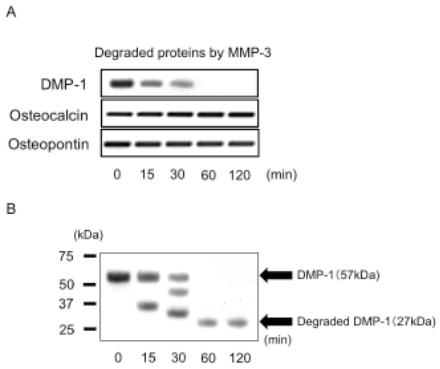

Figure 2. Time-dependent degradation of bone matrix proteins DMP-1, osteocalcin, and osteopontin by MMP-3. (A) Western blotting analysis of proteins incubated with exogenous active MMP-3 and recombinant mouse DMP-1, osteocalcin and osteopontin for 0, 0.25, 0.5, 1, and 2 h using a molar ratio of 1:1 at 37°C, respectively. **(B)** Western blotting analysis of proteins incubated with exogenous active MMP-3 and recombinant mouse DMP-1 with Coomassie brilliant blue staining for 0, 0.25, 0.5, 1, and 2 h using a molar ratio of 1:1 at 37°C. Before Western blot analysis, MMP-3 was immunoprecipitated by incubation with anti-MMP-3 polyclonal antibody and protein A/G Sepharose for 1 h at 4°C. Visualization of blotted protein bands was performed with Multi Gauge-Ver3.X software (Fujifilm). Blots shown are representative of three independent experiments.

DMP-1 were increased in the presence of 0.25 ng/mL and 2.5 ng/mL IL-1β, but not at 25 ng/mL.

To assess whether the induction of DMP-1 by IL-1β is a specific response in ESC-derived odontoblast-like cells, we evaluated the expression of other osteoblastic markers, including osteocalcin and osteopontin proteins, following treatment with the same concentrations of extracellular IL-1β (0, 0.25, 2.5, and 25 ng/mL). However, there were no significant increases in mRNA levels of these markers in response to IL-1β (Figures 1B and 1C). KN-3 odontoblast cells showed similar responses to the ESC-derived odontoblast-like cells (Figures 1D-1F).

3.2. *MMP-3 degraded DMP-1 protein in vitro*

When exogenous active MMP-3 was mixed with recombinant mouse DMP-1, prior to incubation at 37°C for 2 h, the intensity of both DMP-1 protein bands decreased in a time-dependent-manner, as visualized by western blot, indicating that DMP-1 as a dentin matrix component could be the substrate for MMP-3 (Figure 2A). Interestingly, the other bone matrix protein components and osteoblast markers, osteocalcin and osteopontin, showed no differences, suggesting that MMP-3 could not degrade these proteins under similar conditions. In addition, when activated MMP-3 and recombinant DMP-1 were incubated at 37°C, mature DMP-1 (57 kDa) decreased time-dependently, but the degraded product of DMP-1 (27 kDa) appeared for up to 2 h (Figure 2B).

3.3. *Effect of exogenous degraded product of DMP-1 protein by MMP-3 on cell proliferation*

Subsequent experiments used ESC-derived odontoblast-like cells (E14Tg2a) in comparison to rat KN-3 cells. We tested whether exogenous degraded products of DMP-1 by MMP-3 could enhance cell proliferation in odontoblast-like cells. DMP-1 and the active form of MMP-3 were mixed, incubated and added to the anti-MMP-3 polyclonal antibody. Protein A/G sepharose was then added and this was spun down to remove MMP-3. When the supernatant (containing DMP-1 degradation products) was added to odontoblast-like cells, cell proliferation was slightly increased ($p < 0.05$; Figures 3A and 3B). Therefore, we confirmed that the enhanced effect on cell proliferation was dependent on the degraded products of DMP-1 and not exogenous MMP-3 (Figure 3A). We also confirmed that the addition of DMP-1 had no effect on cell proliferation in odontoblast-like cells (Figures 3C and 3D). Therefore, the degraded products of DMP-1 were required for cell proliferation. KN-3 cells showed similar responses to the ESC-derived odontoblast-like cells (Figures 3B and 3D). The supernatant was confirmed to have no MMP-3 activity (Figure 3E). Interestingly, DMP-1 products had no proliferative effect on mouse osteoblastic MC3T3-E1 cells (Figure 3F).

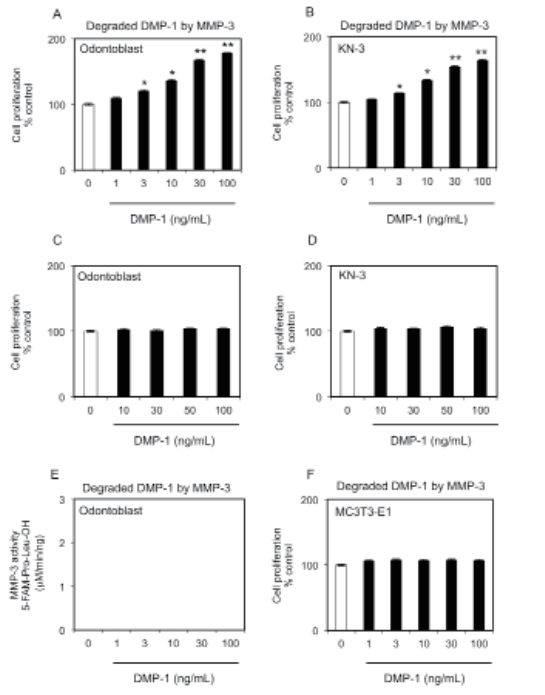

Figure 3. Effect of DMP-1 degradation products by MMP-3 on cell proliferation. Effect of exogenous DMP-1 degradation products by MMP-3 on cell proliferation of ESC-derived odontoblast-like cells **(A)** and KN-3 cells **(B)**. **(C)** Effect of exogenous DMP-1 protein on cell proliferation of ESC-derived odontoblast-like cells **(D)** and KN-3 cells. **(E)** Determination of MMP-3 activity in the supernatant containing DMP-1 degradation products by MMP-3. One unit of MMP-3 activity is defined as 1 μM of 5-FAM-Pro-Leu-OH formed/min/ng enzyme at 37°C. **(F)** Effect of DMP-1 degradation products by MMP-3 on MC3T3-E1 cell proliferation.

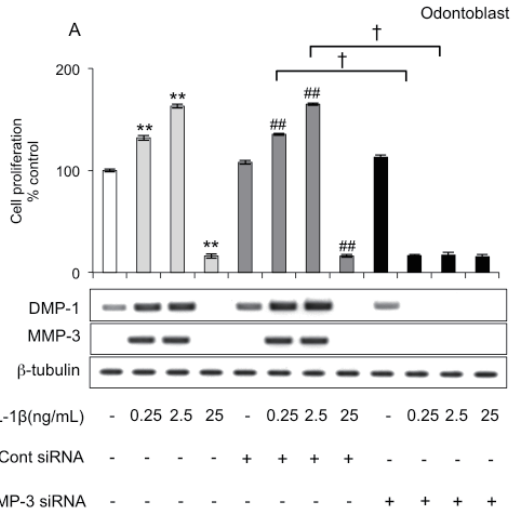

Figure 4. Effect of anti-mouse DMP-1 polyclonal antibody on IL-1β-induced MMP-3 expression and cell proliferation. ESC-derived odontoblast-like cells **(A** and **C)** and KN-3 cells **(B** and **D)** were treated for 24 h with anti-DMP-1 polyclonal antibody, stimulated with IL-1β (0, 0.25, 2.5, and 25 ng/mL), then analyzed by BrdU-cell proliferation ELISA (for cell proliferation; A and B graphs, upper panels), MMP-3 activity **(C** and **D)** and western blotting (for MMP-3 protein expression; **C** and **D**, far lower panels). β-Tubulin was used as a housekeeping protein in the western blots. $^{**}p < 0.01$ *vs*. control; $^{\#\#}p < 0.01$ as indicated by the bracket. Cell proliferation was estimated by BrdU-ELISA. ELISA data represent the mean ± S.D. of four independent experiments. $^{*}p < 0.05$ and $^{**}p < 0.01$.

3.4. *Effect of anti-DMP-1 polyclonal antibody on IL-1β-induced MMP-3 expression and cell proliferation*

Because there is no commercially available mouse DMP-1 siRNA, we employed a specific anti-DMP-1 polyclonal antibody to determine if the proliferation effects observed with IL-1β stimulation were mediated by DMP-1. Cells were pretreated with an anti-DMP-1 polyclonal antibody, and then stimulated with IL-1β as described above. Western blot analysis of MMP-3 protein expression confirmed that the antibody had no effect on the IL-1β-induced MMP-3 activity (Figure 4C). Anti-DMP-1 polyclonal antibody had no effect on the expression of β-tubulin (loading control). Using the same culture conditions, we tested the effect of an anti-DMP-1 polyclonal antibody on IL-1β-induced changes in cell proliferation. DMP-1 silencing considerably decreased the number of proliferating odontoblast-like cells following IL-1β stimulation when compared with untreated cells ($p < 0.01$; Figure 4A). The reduction in proliferative potential was estimated to be ~60% and up to the basal level, suggesting that IL-1β-induced cell proliferation required DMP-1 protein in odontoblast-like cells.

Using the same culture conditions, KN-3 cells showed a similar response to ESC-derived odontoblast-like cells (Figures 4B and 4D).

3.5. *Effect of anti-MMP-3 siRNA on IL-1β-induced DMP-1 expression and cell proliferation*

We replicated the above experiments using anti-MMP-3 siRNA. This reagent efficiently down-regulated the DMP-1 protein expression induced by IL-1β (0.25 and 2.5 ng/mL; $p < 0.01$; Figure 5A). Conversely, the control siRNA had no such effect (Figure 5A). Silencing the MMP-3 gene also significantly decreased IL-1β-stimulated cell proliferation ($p < 0.01$; Figure 5A) as described previously (*9,10*). Taken together, these data suggest that IL-1β-induced DMP-1 expression depends on the expression of MMP-3 in odontoblast-like cells. Using the same culture conditions, KN-3 cells showed similar responses to the ESC-derived odontoblast-like cells (Figure 5B).

3.6. *Effect of anti-Wnt5a and -Wnt5b siRNA on IL-1β-induced DMP-1 expression and cell proliferation*

We replicated the above experiments using anti-Wnt5a and -Wnt5b siRNA. These reagents efficiently down-regulated the DMP-1 protein expression induced by IL-1β (0.25 and 2.5 ng/mL; $p < 0.01$; Figures 6A and 6B). Conversely, the control siRNA had no such effect (Figures 6A and 6B). Silencing the Wnt5 gene also significantly decreased IL-1β-stimulated cell proliferation ($p < 0.01$;

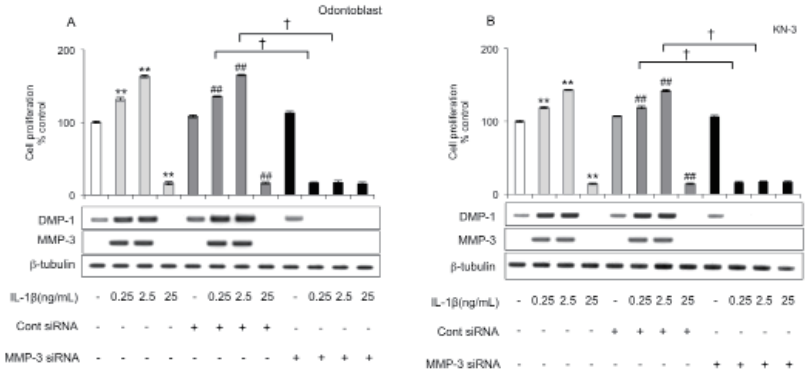

Figure 5. Effect of MMP-3 siRNA on IL-1β-induced DMP-1 expression and cell proliferation. ESC-derived odontoblast-like cells **(A)** and KN-3 cells **(B)** were transfected for 24 h with MMP-3 siRNA, stimulated with IL-1β (0, 0.25, 2.5, and 25 ng/mL), then analyzed by BrdU-cell proliferation ELISA (for cell proliferation) and western blotting (for DMP-1 and β-tubulin protein expression). $^{**}p < 0.01$ *vs*. control; $^{\#\#}p < 0.01$ *vs*. control siRNA; $^{\dagger}p < 0.01$, as indicated by the bracket.

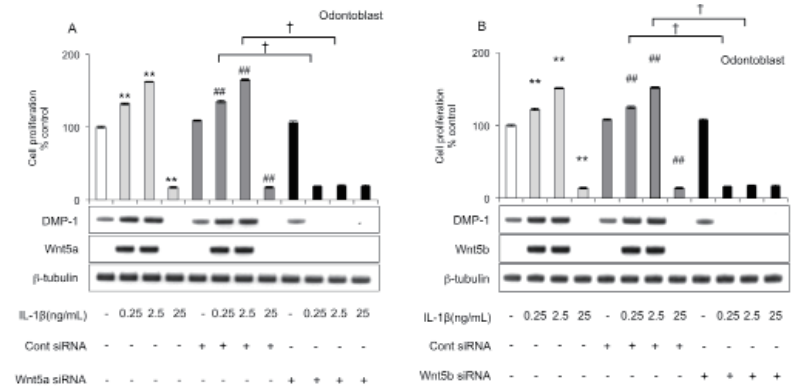

Figure 6. Effect of Wnt5a and Wnt5b siRNA on IL-1β-induced DMP-1 expression and proliferation in ESC-derived odontoblast-like cells. ESC-derived odontoblast-like cells were transfected for 24 h with Wnt5a or Wnt5b siRNA, stimulated with IL-1β (0, 0.25, 2.5, and 25 ng/mL), then analyzed by BrdU-cell proliferation ELISA (for cell proliferation) and western blotting (for DMP-1 protein expression). β-Tubulin was used as a housekeeping protein in the western blots. $^{**}p < 0.01$ *vs*. control; $^{\#\#}p < 0.01$ *vs*. control siRNA; $^{\dagger}p < 0.01$, as indicated by the bracket.

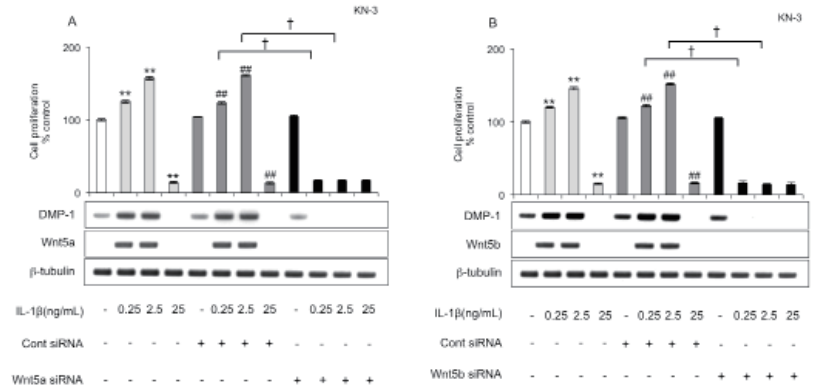

Figure 7. Effect of Wnt5a and Wnt5b siRNA on IL-1β-induced DMP-1 expression and proliferation in KN-3 cells. KN-3 cells were transfected for 24 h with Wnt5a or Wnt5b siRNA, stimulated with IL-1β (0, 0.25, 2.5, and 25 ng/mL), then analyzed by BrdU-cell proliferation ELISA (for cell proliferation) and western blotting (for DMP-1 protein expression). β-Tubulin was used as a housekeeping protein in the western blots. $^{**}p < 0.01$ *vs*. control; $^{\#\#}p < 0.01$ *vs*. control siRNA; $^{\dagger}p < 0.01$, as indicated by the bracket.

Figures 6A and 6B). Taken together, these data suggest that IL-1β-induced DMP-1 expression depends on the expression of Wnt5 in odontoblast-like cells. Using the same culture conditions, KN-3 cells showed similar responses to the ESC-derived odontoblast-like cells ($p < 0.01$; Figures 7A and 7B).

In line with our previous report (*29*), we examined the sequential order in which Wnt5, MMP-3 and DMP-1 are expressed in odontoblast-like cells using western blot analysis and siRNA silencing. This signaling cascade appeared to be IL-1β→Wnt5→MMP-3→DMP-1→DMP-1 degradation products by MMP-3, and intimately involved in the cell proliferation of ESC-derived odontoblast-like cells (Figure 8).

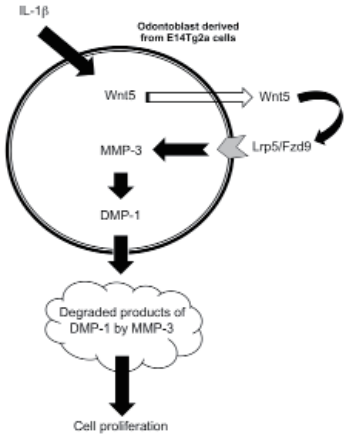

Figure 8. Schematic illustrating the putative signaling pathway by which IL-1β stimulates MMP-3 activity to induce cell proliferation in ESC-derived odontoblast-like cells.

4. Discussion

We have demonstrated previously that the proinflammatory cytokine IL-1β induces MMP-3-regulated cell proliferation in odontoblast-like cells and in KN-3 cells (*9,10*). While recent reports demonstrate that IL-1β-induced MMP-3 is associated with the secreted glycoprotein Wnt signaling pathway (*30,31*), we also confirmed that Wnt5a and Wnt5b are associated with IL-1β-induced proliferation in odontoblast-like cells derived from mouse ESCs (*16*). However, the mechanistic basis and signal cascade, especially downstream of MMP-3 for this action, is unknown.

The monoclonal anti-α2 integrin antibody is known to potently suppress the expression of odontoblastic markers in these culture systems. We have previously confirmed that α2 integrin expression in ESCs triggered their differentiation into odontoblast-like cells (*12*). The proportion of α2 integrin-positive cells in the total population of differentiated odontoblast-like cells is a measure of the purity of the E14Tg2a-derived odontoblast-like cell preparation. Fluorescence-activated cell sorting analysis estimated the proportion of these cells to be 98.77 ± 2.24% (n = 3). In the present study, IL-1β-induced MMP-3 was found to stimulate the degradation of dentin matrix proteins, such as DMP-1, resulting in up-regulation of odontoblast-like cell proliferation by the degraded products. Interestingly, DMP-1 expression was dependent on IL-1β-induced MMP-3 in the odontoblast-like cells. It is therefore conceivable that compensatory increases in DMP-1 production by the odontoblast-like cells, as the counter partner, may occur in some stages during the progression of inflammation (within the microenvironment). Although it is unclear how IL-1β-induced MMP-3 regulates odontoblast-like cell proliferation and which molecular pathways in detail are involved in upregulation of MMP-3 upon exposure to IL-1β, we believe that the mechanism of IL-1β-induced cell proliferation is clearly different from intrinsic cell growth and DMP-1 production. This observation may represent a novel physiological function of MMP-3 and be physiologically relevant in counteracting the effects of local inflammation. Although DMP-1 has previously been reported to enhance cell attachment and migration (*32*), there are no reports on the effect of DMP-1 on cell proliferation, as shown in the current study.

In addition, we found a significant increase of IL-1β-induced MMP-3 in the cells. To extend and invade into dentin containing dentin matrix proteins, it is natural that odontoblasts have to secrete the destructive enzyme MMP-3. We demonstrated that IL-1β-induced MMP-3 could degrade DMP-1, but not the osteoblastic markers osteocalcin and osteopontin (Figure 2). Furthermore, the DMP-1 degradation products caused enhanced cell proliferation in odontoblasts, which may lead to the formation of dentin. We also confirmed that DMP-1 had no effect on cell proliferation in odontoblast-like cells (Figure 3). Although this mechanism is contradictory, an effective and novel autoregulation system for dentin metabolism within a closed microenvironment site, for example at sites of inflammation, is important for cell proliferation.

Our previous report demonstrates that this signaling cascade appears to be IL-1β→Wnt5→Lrp5/Fzd9→MMP-3, and is intimately involved in cell proliferation in stem cell-derived odontoblast-like cells (*33*). Combined with current evidence, we have demonstrated the signal cascade as follows: IL-1β→Wnt5→MMP-3→DMP-1→DMP-1 degradation products by MMP-3→cell proliferation (depicted in Figure 8). Odontoblasts produce and secrete dentin matrix proteins, including DMP-1, and are therefore surrounded by their products (*34*). DMP-1 is proteolytically processed into N- and C-terminal fragments in the dentin ECM and has been identified as a high-molecular-weight proteoglycan comprising the N-terminal DMP-1 fragment and chondroitin sulfate (*34*). Interestingly, because treatment with IL-1β results in the potent induction of DMP-1 protein, this production also may contribute to regeneration and wound healing. In addition to their dentin-secretory activity, odontoblasts play a role in defensive mechanisms and the stimulation of inflammatory responses against pathogen invasion through dentinal tubules (*34*). Although we identified a novel physiological function in mouse odontoblast-like cells in the current study, it remains to be elucidated whether a similar physiological function of human odontoblasts plays an important part against the inflammatory state. Because we have established conditions for the efficient conversion of human muscle stem cells to an odontoblast lineage (*35*), we will next examine these concerns.

Although we tried to identify the peptide sequences with proliferative action derived from DMP-1 by MMP-

3, we could not determine anything specific because of the numerous degradation products derived from this protein. We will therefore attempt to identify the peptide sequence(s) with proliferative action in the near future. When activated MMP-3 and recombinant DMP-1 were incubated at 37°C, mature DMP-1 (57 kDa) decreased time-dependently, while the degradation product of DMP-1 (27 kDa) appeared for up to 2 h. We therefore speculated that this degradation product of DMP-1 contributed to the cell proliferation of odontoblast-like cells (Figure 2B). Because DMP-1 belongs to the SIBLING family (*36*), it is speculated that the RGD sequence may be essential for cell proliferation. Dentin sialophosphoprotein (DSPP) and DMP-1 share many similarities in both their gene and protein structures, and it is now believed that DSPP arose from DMP-1 by a gene duplication event (*37*). DSPP and DMP-1 are both cleaved into two protein chains; the N-terminal regions are proteoglycans that contain chondroitin sulfate chains, and the C-terminal regions are highly phosphorylated (*38-40*). Because it is now obviously accepted that DSPP and DMP-1 play important roles in hard tissue development, DSPP and DMP-1 are positive regulators of hard tissue mineralization, with both acting on dentin. From preliminary studies, we have confirmed that IL-1β-induced DSPP elicits similar responses in odontoblast-like cells (data not shown), suggesting that the common mechanism between DMP-1 and DSPP may promote the proliferative effect on odontoblasts. The signal cascade, especially the downstream DMP-1 degradation products by MMP-3, remains to be elucidated.

A blocking experiment was carried out using polyclonal antibodies, which showed inhibition of cell proliferation (Figure 4). Because the polyclonal antibodies recognized every open region conformation and did not react with the closed region of DMP-1, one question that arises from this experiment is why intact DMP-1 had no proliferation activity (Figures 3C and 3D), which remains to be elucidated. Another concern of the present study is that IL-1β-induced MMPs, except for MMP-3, are required for cell proliferation. A peptide generated through the proteolytic processing of DMP-1 by MMP-2 can regulate the differentiation of mesenchymal cells during dentinogenesis and thus sustain reparative dentin formation in pathological situations, such as carious decay (*41*). However, we confirmed that there was no significant increase in MMP-2 mRNA in response to IL-1β-induced odontoblast-like cells derived from ESCs and KN-3 cells (data not shown).

In summary, increased MMP-3 activity may contribute not only as a primary initiating trigger for the destruction for bone matrix components, but also as a compensatory response owing to the degradation of dentin matrix protein, thus promoting cell proliferation, leading to formation of dentin. We have demonstrated that DMP-1 responds to IL-1β by up-regulating MMP-3 expression *via* the Wnt5 signaling pathway in mouse ESC-derived odontoblast-like cells. These results provide new insights into the role of MMP-3 in odontoblast cells, and may have relevance to our understanding of and ability to improve wound healing following dental pulp injury.

Acknowledgements

This work was supported by a Grant-in-Aid-for Scientific Research (A) (Grant No. 25253101; to SY), a Grant-in-Aid-for Scientific Research (C) (Grant No. 26462904; to NO) and a Grant-in-Aid-for Young Scientists (B) (Grant No. 15K20418; to NH) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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(Received July 7, 2015; Revised August 8, 2015; Accepted August 9, 2015)